Osteoarthritis and Cartilage 21 (2013) 965–972

Osteoarthritis and Cartilage

OARSI OSTEOARTHRITIS RESEARCH SOCIETY INTERNATIONAL

Gradual strenuous running regimen predisposes to osteoarthritis due to cartilage cell death and altered levels of glycosaminoglycans

C.E.S. Franciozi †*, V.A.F. Tarini †, R.D. Reginato ‡, P.R.S. Gonçalves ‡, V.P. Medeiros §, M. Ferretti †, J.L. Dreyfuss §, H.B. Nader §, F. Faloppa †

† Department of Orthopaedics and Traumatology, Escola Paulista de Medicina, Universidade Federal de São Paulo, São Paulo, SP, Brazil
‡ Department of Morphology and Genetics, Escola Paulista de Medicina, Universidade Federal de São Paulo, São Paulo, SP, Brazil
§ Molecular Biology Program, Escola Paulista de Medicina, Universidade Federal de São Paulo, São Paulo, SP, Brazil

ARTICLE INFO

Article history:
Received 21 December 2012
Accepted 6 April 2013

Keywords:
Articular cartilage
Extracellular matrix
Apoptosis
Running
Chondroitin sulfate
Hyaluronic acid

SUMMARY

Objective: To investigate the hypothesis that strenuous running is a predisposing factor for osteoarthritis.
Design: Wistar rats were divided into two groups: a control group (CG) and a trained group (TG). The TG underwent a strenuous treadmill running training regimen of controlled intensity, exhibiting progressively improvement of fitness over 12 weeks, running at least 55 km during this period and finally performing an ultra-endurance running exercise to exhaustion. After this period, rats from both groups were euthanized and their knees removed. The articular cartilage was dissected and submitted to histomorphometrical, histomorphological, and immunohistochemical analyses evaluating cell death pathway (caspase-3 and terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL)) and inflammatory cytokines [interleukin-1α (IL-1α) and tumor necrosis factor-α (TNF-α)]. In addition, the tissues were analyzed regarding the types and the content of glycosaminoglycans.
Results: The TG knee joints exhibited increase in the number of chondrocytes and chondrocyte clusters, as well as significantly increased levels of caspase-3, a protein involved in apoptosis, and of inflammatory cytokines IL-1α and TNF-α. In addition, histologically higher grades of osteoarthritis (Osteoarthritis Research Society International – OARSI grading), and significantly decreased levels of chondroitin sulfate and hyaluronic acid. Knee cartilage thickness and TUNEL did not significantly differ between the two groups.
Conclusions: The articular cartilage of rats subjected to a strenuous running regimen of controlled intensity exhibited molecular and histological characteristics that are present in osteoarthritis.

Introduction

The beneficial effects of a lifestyle that involves physical exercise are well established, and physical inactivity is one of the three leading causes of preventable morbidity, mortality and disability in developed countries[1]. Increasing physical activity levels of the population is a constant objective of government policy and private investment despite the lack of studies evaluating these interventions[2].

Articular cartilage can be affected by physical activity. Its complex composition provides a lubricated surface that absorbs and transfers load to allow joint movement without friction[3]. The development, maintenance, and destruction of cartilage are regulated by mechanical factors throughout life[4]. The microenvironment of the articular cartilage plays important role in its biomechanical function. The properties of this tissue are related to the composition of its extracellular matrix, mainly composed of proteoglycans and hyaluronic acid (HA) entangled in a dense network of collagen fibers that retain large amounts of water. The structures of these molecules play major roles in determining the resilience of the tissue to compression[5–7]. Proteoglycans in articular cartilage form large aggregates that consist of a central HA filament to which multiple monomers are non-covalently attached[5].

Chondrocytes are the only cell type of the articular cartilage. They maintain tissue homeostasis, react to injury and carry out cartilage remodeling. Chondrocyte death and survival are related to cartilage

* Address correspondence and reprint requests to: C.E.S. Franciozi, Department of Orthopaedics and Traumatology, Escola Paulista de Medicina, Universidade Federal de São Paulo, Rua Borges Lagoa, 783–5°Andar, Vila Clementino, 04038-032 São Paulo, SP, Brazil. Tel: 55-11-5571-6621.
E-mail addresses: cacarlos66@hotmail.com (C.E.S. Franciozi), victor.tarini@gmail.com (V.A.F. Tarini), rejane.dr@uol.com.br (R.D. Reginato), patriciareissgoncalves@yahoo.com.br (P.R.S. Gonçalves), valmedeiros@gmail.com (V.P. Medeiros), marioferretti@uol.com.br (M. Ferretti), jdreyfuss@unifesp.br (J.L. Dreyfuss), hbnader.bioq@epm.br (H.B. Nader), faloppa.dot@gmail.com (F. Faloppa).

1063-4584/$ – see front matter
http://dx.doi.org/10.1016/j.joca.2013.04.007

matrix integrity. Apoptosis and necrosis are mechanistically and morphologically distinct types of cell death[8]. Apoptosis is a form of programmed cell death, and a singular pattern of apoptotic cell death is the activation of caspases. Although not all forms of apoptosis depend on caspases, these proteases are key factors in the initiation and the execution of the process. Pro-inflammatory cytokines such as interleukin-1α (IL-1α) and tumor necrosis factor-α (TNF-α) induce matrix metalloproteinases and aggrecanases which generate matrix degradation products that can contribute to the activation of catabolic responses in chondrocytes[9,10].

The effects of exercise on articular cartilage remain a subject of debate and speculation, as does the relationship of exercise to the development of osteoarthritis. Moderate exercise benefits this tissue[11], but consensus is lacking regarding deleterious effects of heavy physical activity, elite level competition, and long-distance or marathon running, leaving unanswered the question: is strenuous running a predisposing cause of osteoarthritis?[11–14]

The present study intends to answer that question and the results confirm the hypothesis that strenuous running is a predisposing cause of osteoarthritis.

Methods

Animals

Twenty-eight albino Wistar male rats 15–20 weeks old at the beginning of the experiment were housed at 22°C with a light–dark cycle (7:00 am–7:00 pm). The animals were allowed access to food and water *ad libitum*.

Ultra-endurance exercise training

Ultra-endurance exercise is defined as running a distance of at least 50 km or for more than 4 h[15,16]. To induce this condition in experimental studies, a novel protocol was devised according to the Animal Research: Reporting In Vivo Experiments (ARRIVE) guidelines[17,18]. A motorized treadmill (AVS Projetos, São Paulo, SP, Brazil) with six individual lanes and no inclination was used. A shock grid at the back of the treadmill delivered a mild shock (2.0 mA) if the rat's pace fell below the treadmill speed. All rats were adapted to walking on the treadmill for 3 days at 10 m/min for10 min per day. To assess trainability, each animal's performance was rated on a scale of 1–5 according to Dishman *et al.*[19]. Animals with a mean rating of 3 or higher were included in the study and those with a mean rating of 1 or 2 were excluded to avoid differentially stressed animals in the study. Four animals were excluded due to insufficient score.

Animals were divided into two experimental groups of 12 individuals according to Dishman's score: control group (CG) and trained group (TG). The animals from the CG did not perform any exercise after the division into groups. The TG was subjected to a progressive endurance regimen 5 days/week for 12 weeks. The initial velocity of 10 m/min was increased to 15 m/min at the third week, 20 m/min at the fifth week, and at least 25 m/min at the seventh week and thereafter. The duration of the sessions was increased by 10 min every 2 weeks starting at the second week to reach 70 min per session at 12 weeks of training. Top speed tests were performed every 30 days to correct the intensity of the exercise as the animals adapted to the progressive training. The top speed test consisted of running the rats at an initial velocity of 10 m/min. The speed was increased 2 m/min every 3 min until exhaustion. The top speed was designated as that recorded 3 min prior to the point of exhaustion. Since the need to correct and control intensity training, each rat ran at 60% its top speed; this intensity was used throughout the study[17].

After 12 weeks, animals in the TG that had run approximately 55 km underwent an ultra-endurance exercise on a treadmill at an intensity corresponding to 60% top speed until exhaustion. Afterward, the animals of both groups were sacrificed by decapitation under sedation. Femoral condyles from the right and left hind legs were carefully dissected without damaging the cartilage surface.

Histology and histochemistry

The right distal femoral condyles were fixed at room temperature for four days in 4% formaldehyde buffered at pH 7.2 with 0.1 M sodium phosphate and were decalcified for 30 days in 25% formic acid, pH 2.0. Specimens were dehydrated in graded concentrations of ethanol and embedded in paraffin. Serial 5 μm coronal sections were subjected to histomorphometry, immunohistochemistry, and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay.

Sections were stained by hematoxylin and eosin (HE) and safranin O. HE sections were subjected to histomorphometric analysis performed with a semiautomatic image analysis system (AxioVision Rel. 4.6., Carl Zeiss, Oberkochen, Germany). To evaluate the thickness of the articular cartilage, images were captured with a 2.5× objective lens. To count the number of chondrocytes and the clusters of chondrocytes, the images were captured with a 10× objective lens. Three different regions of the articular cartilage at both the weight-bearing and non-weight-bearing areas of the femoral condyles were defined by a rectangle with a standardized area of approximately 10,000 μm^2.

Safranin O sections were evaluated according to the *Osteoarthritis Research Society International* (OARSI) grading system for articular cartilage degeneration[20].

Staining of active caspase-3, IL-1α, TNF-α and HA was performed by indirect immunohistochemical or histochemical analyses using anti-caspase-3, anti-IL-1α, anti-TNF-α (all from Biocare Medical, Concord, CA) as the primary antibodies or biotinylated HA binding protein all diluted 1:100 and incubation on the sections for 1 h. Next, streptavidin-labeled secondary antibodies or streptavidin-horseradish peroxidase (HRP) were applied and then developed with 0.05% diaminobenzidine tetrachloride. HE was used to counterstain. The primary antibodies were replaced with phosphate-buffered saline to prepare negative controls; no detectable staining was evident. Images of chondrocytes in the superficial (SZs) and intermediate (IZs) zones of both the weight and non-weight-bearing areas of the femoral condyles were captured with a 40× objective lens, and those cells within a predetermined rectangular area of 50.56 × 114.78 μm^2 were counted. The proportion of chondrocytes staining for caspase-3, IL-1α, and TNF-α was calculated and expressed as a percentage of the total number of cells within each area. Data were expressed in mean with lower and upper limits of 95% confidence intervals (CIs).

TUNEL assay

Apoptotic cells were detected *in situ* using a TUNEL assay according the manufacturer's instructions (*ApopTag® Peroxidase*, Chemicon, Temecula, CA, USA). TUNEL-positive hypertrophic chondrocytes were observed at the growth plates, serving as an internal positive control for each section. For negative controls, sections were incubated with label solution alone (without terminal transferase or TUNEL reaction mixture) (see Supplementary Data). Positive chondrocytes were counted and the proportion of TUNEL staining was expressed as the percentage of the total cells in each area. Data were expressed as mean with lower and upper limits of 95% CI.

Extraction, isolation, and quantification of glycosaminoglycans (GAGs)

GAGs were extracted from the cartilage of the left femur samples after careful dissection from the bone. The cartilage samples were grinded and dehydrated in acetone for 24 h. The dried samples were weighted and incubated for 18 h at 60°C in 0.1 M phosphate buffer-cysteine, pH 6.5, containing 2 mg/ml papain (Calbiochem, Darmstadt, Germany) and 0.02 M Ethylenediamine tetraacetic acid (EDTA). Afterward, peptides and nucleic acid fragments were removed by precipitation with trichloroacetic acid (10% final concentration) at 4°C. After centrifugation (10 min, 3,500× *g*, 4°C), the supernatant containing GAGs was precipitated by adding five volumes of methanol for 18 h at −20°C. The precipitate was collected by centrifugation and dried. Recovery of GAGs extracted from cartilage by this method is approximately 95%[21,22]. The samples were then subjected to chemical β-elimination by treatment with 0.05 M sodium borohydride, 0.05 M NaOH overnight at room temperature to release GAGs chains from the remaining peptides of the core proteins. The samples were neutralized with 10% acetic acid, dialyzed against distilled water for 4 h at 4°C, and lyophilized, suspended in distilled water (5 mg of dry tissue to 10 ml) and analyzed for sulfated GAGs and HA content. GAGs were identified and quantified by agarose gel electrophoresis in 0.05 M 1,3-diaminopropane acetate buffer, pH 9.0[23]. The sulfated GAGs were identified comparing their migration to chondroitin sulfate (CS), dermatan sulfate (DS), and heparan sulfate (HS) standards. The samples were analyzed in triplicate. The quantitative results were expressed as μg sulfated GAGs per g of dry tissue. The values were expressed as means with lower and upper limits of 95% CIs.

Determination of HA was performed using a highly specific enzyme-linked immunosorbent assay (ELISA)-like fluorimetric method. The samples obtained as described above were boiled for 30 min in order to inactivate any proteolytic activity. Afterward they were dried and suspended in distilled water (5 mg of dry tissue to 10 ml). A 5 μl aliquot of each sample was diluted 1:1,000 with 0.05 M Tris–HCl, pH 7.8, and triplicate 100 μl samples of this dilution were analyzed for HA content. The absolute amounts of HA were expressed as micrograms of HA per gram of dry tissue. The values were expressed as means with lower and upper limits of 95% CIs[22].

Statistical analyses

The distribution of the quantitative data in this work was assessed using the Kolmogorov–Smirnov test; no distribution strays from the normal distribution ($P > 0.05$). The CG and TG group data values were compared using Student's *t*-test. OARSI classifications were described as absolute and relative frequencies within each group and assessed using the Mann–Whitney test. The data values obtained in histochemical analyses were expressed as the mean of percent positive cells and assessed using the Mann–Whitney test. The Spearman correlation was calculated between OARSI and the histochemical analyses. All statistical tests were considered significant at the level of 5%.

Results

Strenuous running increases the number of chondrocytes

HE-staining to determine the number of chondrocytes present was performed within two main, distinct sites of the articular cartilage: the non-weight-bearing and the weight-bearing areas of the femoral condyles.

Within the SZs and IZs of the non-weight-bearing area, the TG exhibited greater number of chondrocytes [mean 32.58 (95% CI 30.32–34.84)] compared to the CG [mean 24.41 (95% CI 21.52–27.3)]. This difference was statistically significant ($P < 0.001$) [Fig. 1(A)]. Within the deep zone (DZ) of the non-weight-bearing area, the TG exhibited a greater number of chondrocytes [mean 21.67 (95% CI 19.98–23.36)] compared to the CG [mean 20.5 (95% CI 18.52–22.48)], but this difference was not statistically significant ($P = 0.390$) [Fig. 1(A, B)].

Within the SZ and IZ of the weight-bearing area, the TG exhibited a significantly greater number of chondrocytes [mean 30.83 (95% CI 26.49–35.17)] compared to the CG [mean 24.75 (95% CI 21.75–27.75)] and this was statistically significant ($P = 0.034$). Within the DZ of the weight-bearing area, the TG exhibited a statistically significant greater number of chondrocytes [mean 7.67 (95% CI 6.93–8.41)] compared to the CG [mean 5.67 (95% CI 4.97–6.37)] ($P = 0.001$). These results demonstrate a stress response and an attempt of the tissues of animals from the TG that is most pronounced in the weight-bearing area [Fig. 1(A, B)].

Chondrocyte clusters, characteristic of osteoarthritis, were evaluated in the SZs and IZs of the weight-bearing area. The number of chondrocyte clusters was significantly higher in the TG [mean 6.75 (95% CI 6.2–7.3)] compared to the CG [mean 4.58 (95% CI 3.69–5.47)] ($P < 0.001$), indicating that the training regimen for the animals in the TG altered the proliferation pattern [Fig. 1(C, D)].

The thickness of the articular cartilage was evaluated because its reduction is a prominent marker of advanced osteoarthritis. However, although the articular cartilage was thinner in the TG [mean 225.81 μm (95% CI 183.58–268.04)] than in the CG [mean 249.02 μm (95% CI 214.49–283.55)], this difference was not statistically significant ($P = 0.413$).

Strenuous running increases grading for articular cartilage degeneration

Most CG sections were classified according to OARSI as grade 0 and some as grade 1. Most TG sections were classified as grade 2 based on fibrillation through SZ and cationic stain matrix depletion upper 1/3 of cartilage. Some TG sections were also classified as grade 1 and grade 3. No TG sections were graded 0 and no CG sections were graded 2 or 3. Grade 3 was the highest classification given in this study and it was only found in animals of the TG [Fig. 2(A, B)].

Strenuous running leads to cartilage inflammation and cell death detected by histochemical analysis

The process of apoptotic cell death was evaluated using an anti-caspase-3 antibody and the TUNEL assay. The TG exhibited greater percentage of caspase-3-positive cells compared to the CG, mainly in the weight-bearing area [mean 66.12 (95% CI 47.9–84.3)] vs [mean 16.68 (95% CI 8.9–24.4)] ($P = 0.002$), respectively, as well in the non-weight-bearing area [mean 21.55 (95% CI 13.9–29.2) vs mean 7.48 (95% CI 3.7–11.3)] ($P = 0.01$) [Fig. 3].

Apoptotic cell death assessed by the TUNEL assay was not statistically different in the weight-bearing and non-weight bearing areas of the TG compared to CG [Fig. 3].

Inflammatory cytokines (IL-1α and TNF-α) levels were investigated by histochemistry. The TG compared to the CG showed increased percentage of IL-1α-positive cells in the weight-bearing area [mean 62.92 (95% CI 50.1–75.7) vs mean 31.75 (95% CI 29.9–33.6)] ($P < 0.001$) and in the non-weight-bearing area [mean 56.72 (95% CI 54.8–58.7) vs mean 16.43 (95% CI 12.9–19.9)] ($P < 0.001$). In addition, the TG had an elevated percentage of TNF-α-positive cells in the weight-bearing area [mean 77.65 (95% CI 77.3–78) vs

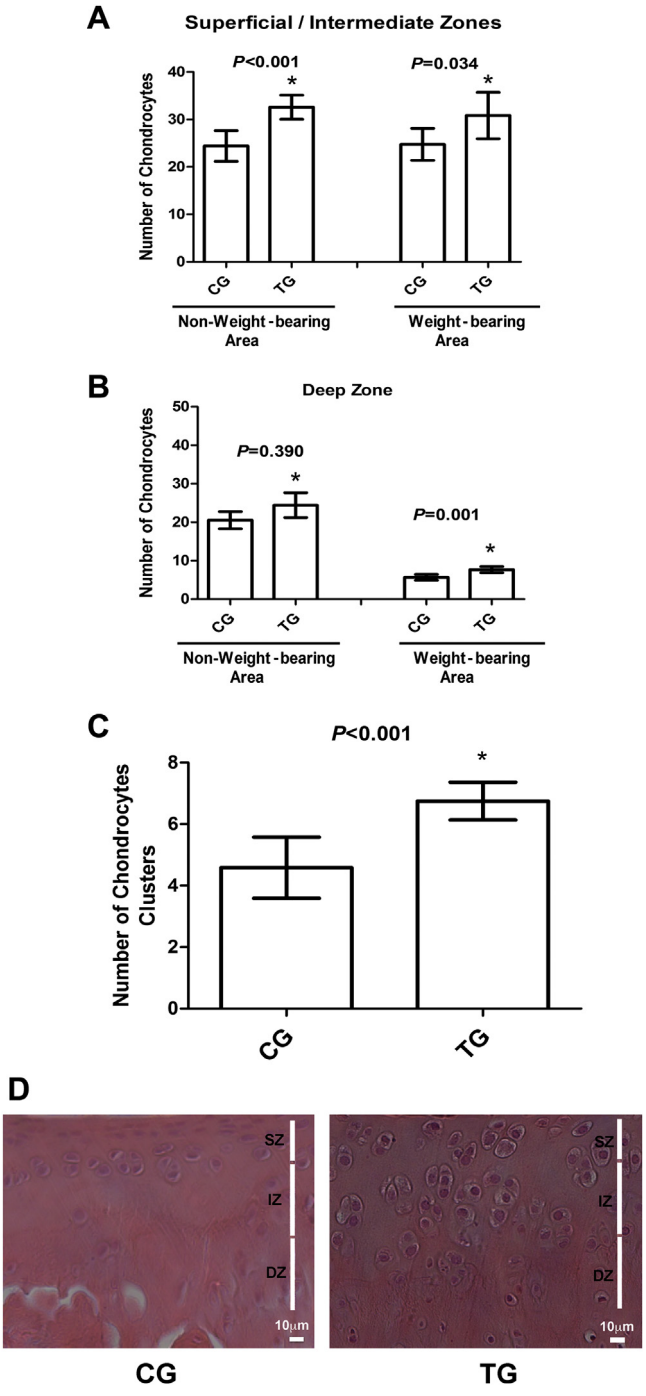

Fig. 1. Histomorphometric and histological analyses of the articular cartilage of 12 CG and 12 TG. (A) Number of chondrocytes in the SZs and IZs of the non-weight-bearing and weight-bearing areas of the articular cartilage. (B) Number of chondrocytes in the DZ of the non-weight-bearing and weight-bearing area of the articular cartilage. (C) Number of chondrocyte clusters in the SZs and IZs of the weight-bearing area of the articular cartilage. Data are expressed in mean with lower and upper limits of 95% CIs and *P* values represents statistical analyses (Student's *t*-test). Star means statistically different results. (D) Representative histological samples of CG and TG stained with HE in a zone-stratified cartilage diagram. Magnification: 400×. Scale bar = 10 μm.

mean 35 (95% CI 32–38)] ($P < 0.001$) and in the non-weight-bearing area [mean 54.4 (95% CI 54–54.8) vs mean 30 (95% CI 24.1–35.9)] ($P < 0.001$) compared to the CG [Fig. 3].

A correlation analysis was made between the observed OARSI grades and the cartilage inflammation and cell death detected by

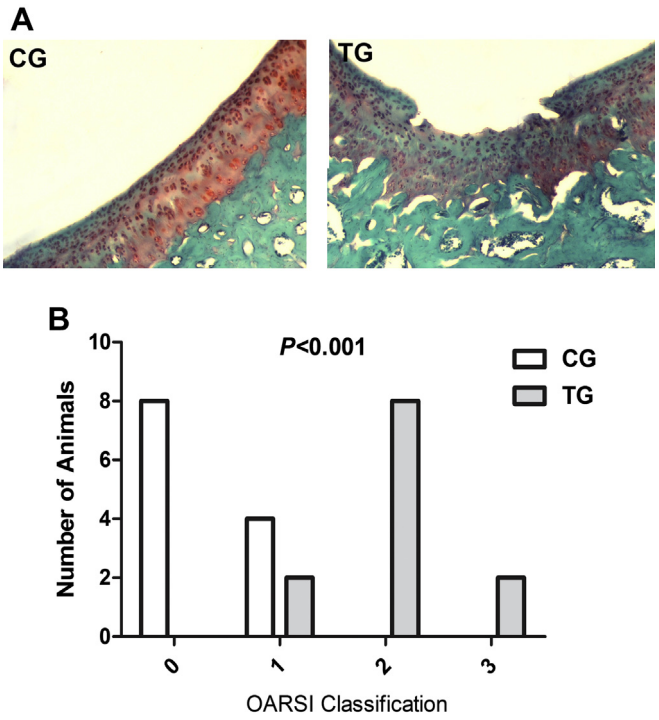

Fig. 2. (A) Representative histological samples CG and TG stained with safranin O. Magnification: 400×. Scale bar = 30 μm. (B) OARSI histological classification of osteoarthritis in the articular cartilage of 12 CG and 12 TG. *P* value represents the results of statistical analyses (Mann–Whitney test) of the absolute and relative frequencies of each grade observed in the CG and TG.

histochemical analysis. Higher OARSI grades were statistically related to higher levels of caspase-3 in the weight-bearing area, IL-1α and TNF-α both in the weight-bearing and non-weight-bearing area. This correlation was not significant in TUNEL analysis of the both areas (Table I). These results show clear association between OARSI grade and apoptosis or OARSI grade and inflammatory molecules. Higher OARSI grades presented significantly higher percentage of positively stained chondrocytes for caspase-3, IL1-α and TNF-α.

Strenuous running decreases GAGs content in articular cartilage

The TG samples contained significantly lower amounts of sulfated GAGs [mean 0.52 μg CS per g of dry tissue (95% CI 0.44–0.6)] than the CG samples [mean 1.33 μg CS per g of dry tissue (95% CI 1.1–1.56)] ($P < 0.001$) [Fig. 4(A, B)]. CS was the main sulfated GAG present in the cartilage of both CG and TG groups [Fig. 4(B)].

Heavy exercise also led to a decrease in the amounts of HA in articular cartilage. HA content in the TG [mean 208.77 μg HA per g of dry tissue (95% 141.45–276.09)] compared to the CG [mean 435.01 μg HA per g of dry tissue (95% 306.07–563.95)] was significantly lower ($P = 0.016$) [Fig. 5 (A)]. Moreover HA in cartilage was also evaluated by histochemistry. The sections from cartilages of animals from the TG showed decreased staining for HA especially in the weight-bearing area [Fig. 5(B)].

These results clearly show significant reduction of both non-sulfated and sulfated GAGs in cartilages of animals from the TG.

Discussion

Light to moderate exercise and mechanical stimuli protect the articular cartilage, increasing the amount of GAGs, reducing and

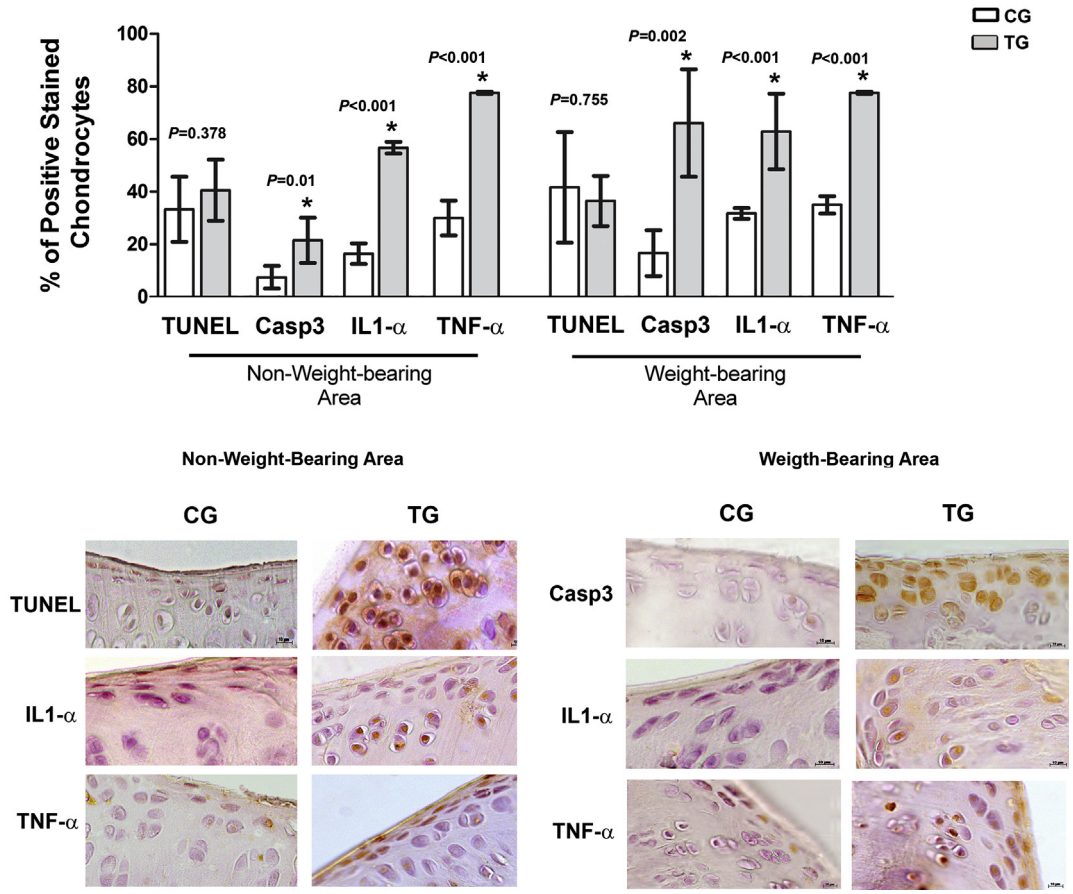

Fig. 3. Immunohistochemical analyses of the articular cartilage of 12 TG and 12 CG animals in weight-bearing and non-weight-bearing areas. The upper panel shows the percentage of positively stained cells for TUNEL assay and caspase-3, IL1-α and TNF-α antibodies. Data are expressed as mean with lower and upper limits of 95% CIs and *P* values represents statistical analyses (Mann–Whitney test). Star means statistically different results. The lower panel shows representative histochemical analyses of articular cartilage sections of CG and TG. Magnification: 400×. Scale bar = 10 μm.

delaying the process of cell death and degeneration, as well as reducing the activity of inflammatory cytokines[11,13]. This protective effect is exploited in the treatment and prevention of osteoarthritis[24]. The question that still remains in the literature concerns excessive exercise or strenuous running and its consequences for the cartilage. Contradictory studies show absence[14,25] or presence[12,26–28] of relationship between strenuous exercise or higher physical activity levels and osteoarthritis. Our results demonstrated a significant decrease of GAG content in the articular cartilage and increased cell death and inflammation after heavy exercise.

The novel protocol of ultra-endurance training in rats developed in this work was intended to evaluate the effects of excessive exercise in healthy joint cartilage[17]; most rat models of osteoarthritis induction employ a surgical procedure that alters the articular harmony[29–31]. A previous model of strenuous running developed by PAP *et al.*[29] involved intracranial stimulation of the rats, whereas TANG *et al.*[32] and SEKIYA *et al.*[33] introduced a modified protocol that motivated the animals by external electrical stimulation of the tail. In both studies, the animals ran 30 km in 6 weeks on a treadmill with 5% inclination. Distal femoral articular cartilages from rats were assessed to determine degeneration since it is an appropriate site to verify the weight-bearing and non weight-bearing areas. The whole-joint cartilage was not assessed; since according to the current literature it is sufficient to monitor specific sites[34].

In contrast to our observations, striking features of osteoarthritis, including erosion with cartilage loss that corresponded to degree 4 of the OARSI histological classification, were observed in those studies. Even though the animals ran nearly double the distance over a period twice as long in our study, the most common OARSI grade observed was 2, which corresponds to fibrillation through the SZ of the articular cartilage. The difference in results could be explained by the use of the 5% inclination in previous studies vs no inclination here together with the gradual improvement in physical fitness generated by our protocol that consisted of 13 km running during the first 6 weeks and 42 km during the last 6 weeks of the experiment. This gradual increase in the excercise was intended to mimic the progressively evolving capacity of strenuous runners allowing for normal adaptation of the muscles, skeletal and cartilage tissue[17].

Table I
Correlation between OARSI classification, inflammation and apoptosis

Variable	Total		
	OARSI correlation	*P*	*N*
TUNEL WB	−0.28	0.186	24
TUNEL NWB	0.061	0.778	24
Caspase-3 WB	0.849	**<0.001**	24
Caspase-3 NWB	0.382	0.065	24
IL-1α WB	0.805	**<0.001**	24
IL-1α NWB	0.805	**<0.001**	24
TNF-α WB	0.728	**<0.001**	24

WB, weight-bearing area; NWB, non-weight bearing area. Bold values represent statistically different results.

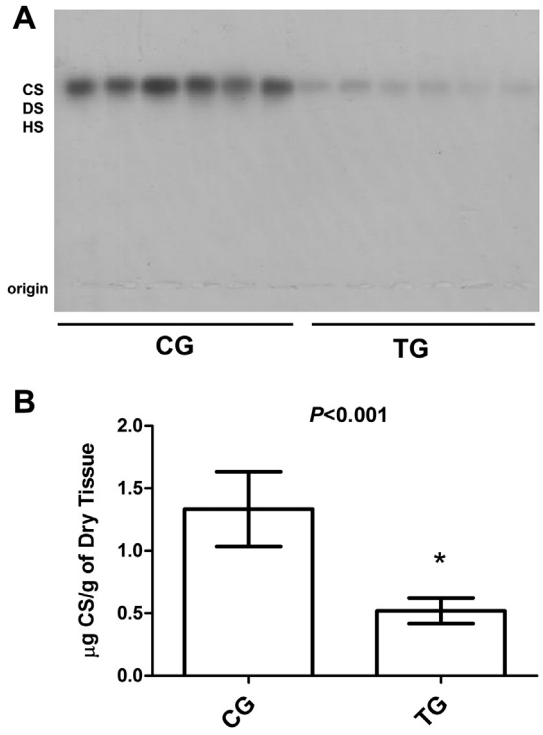

Fig. 4. Analysis of sulfated GAG content extracted from the articular cartilage of CG and TG. (A) A representative agarose gel electrophoresis stained with toluidine blue of GAGs isolated from CG and TG. (B) Quantification of sulfated GAGs in 12 CG and 12 TG. The values are expressed as μg CS per g of dry tissue. Data are expressed as mean with lower and upper limits of 95% CIs and *P* values represents statistical analyses (Student's *t*-test). Star means statistically different results.

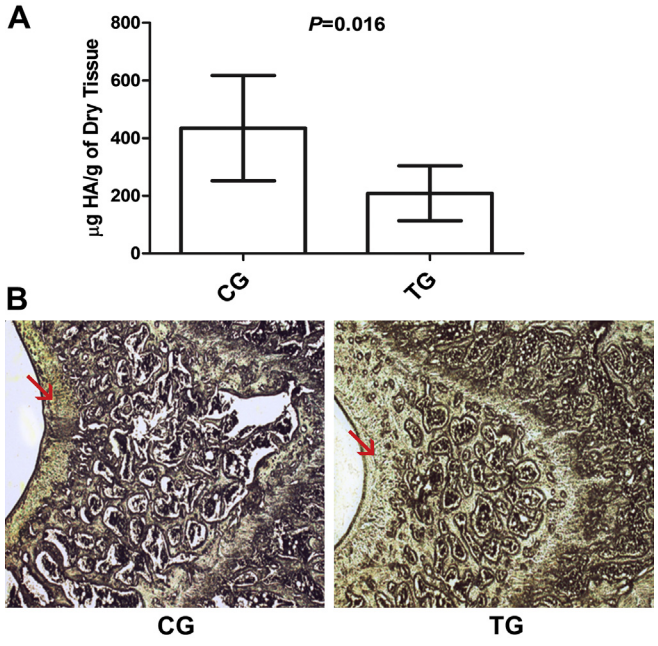

Fig. 5. Analysis of HA content in the articular cartilage of 12 CG and 12 TG. (A) Absolute amounts of HA content in CG and TG. The values are expressed as μg HA per g of dry tissue. Data are expressed as mean with lower and upper limits of 95% CIs and *P* values represent statistical analyses (Student's *t*-test). Star means statistically different results. (B) Representative histochemical analyses of cartilage sections stained for HA of CG and TG. Red arrows indicate the articular cartilage. Magnification: 25×.

Cartilage thickness did not differ significantly between the TG and CG, in contrast to the aforementioned studies[29,32,33]. A response of the cartilage to exercise was demonstrated by increased numbers of chondrocytes and cell death in the TG, in both the weight and non-weight-bearing areas except for the DZ. Increased cell number could be related to tissue repair in response to stress, since chondrocytes can react to mechanical forces and structural changes in the extracellular matrix[3]. The increased number of chondrocytes and cell death in all studied areas of TG demonstrates a tissue response to micro and macro biomechanical changes in the extracellular matrix. These changes include numerous signaling mechanisms that affect the stressed area of the cartilage as well as more distant regions not directly subjected to mechanical stimulation[35,36]. The increase in the number of chondrocyte clusters resulting from excessive exercise demonstrates an acute or sustained proliferation. The formation and growth of clusters is an abnormal cartilage characteristic present in conditions such as osteoarthritis and is considered one of its major phenotypic characteristics[37].

The 2 and 3 OARSI grades observed only in the TG means augmented cartilage depletion and may be related to excessive exercise. In addition, the increased numbers of cells expressing caspase-3, IL-α, and TNF-α in the TG indicate inflammation and apoptosis, which correlate to the observed grade. Cell death can be induced by mechanical stress, changes in the extracellular matrix and in the levels of cytokines and growth factors, among other causes[9,38]. The increase in cell death and decrease in HA and sulfated GAGs content observed in the TG are initial degenerative changes and an early sign of an imbalance favoring catabolism in cartilage functions[3,39,40].

Cell death in osteoarthritis has received substantial consideration in the literature. The ultra-endurance exercise training protocol resulted in clearly increased cell death in the TG compared to the CG. The increase in IL-1α and TNF-α are related to inflammation and the caspase-3 assay indicates cell death process by apoptosis. These pro-inflammatory cytokines can damage the cartilage by inducing the expression of metalloproteinases and other cartilage degrading enzymes, such as aggrecanases[41–44].

Strenuous training lead to increased cell death in cartilage. In this study the apoptotic pathway was evaluated by analyzing caspase-3 immunostaining and TUNEL assay. Caspase-3 assay demonstrated a significant increase in positively stained cells in the weight-bearing and non-weight-bearing areas of the TG. TUNEL assay did not find statistically significant differences between groups. It is known that the TUNEL assay is prone to technical errors, that could explain our results[45].

Regarding extracellular matrix components of articular cartilage, CS was the predominant sulfated GAG present in the articular cartilage of both groups, and the results showed an important decrease in its content for the TG compared to CG. The incident forces on the cartilage affect the balance between anabolism and catabolism. Altered mechanical transduction by the chondrocytes after a loss of intrinsic strength of the SZ changes the mechanical properties of cartilage and is linked to tissue degeneration. GAG depletion decreases compression resistance, which increases chondrocyte compressive loading and upregulates mechanochemical transduction processes that increase expression of catabolic enzymes[46].

Studies showed that decreased GAGs can trigger increased caspase-3 activity, suggesting that compromised matrix can induce chondrocyte apoptosis[39,45,47,48], in accordance to the data presented here where decreased GAG content and augmented caspase-3 detection were observed in the TG. The earliest response to abnormal cell death is acute inflammatory response. In this

process it is observed the production of pro-inflammatory mediators such as IL-1α- and TNF-α. Increased levels of IL-1α- and TNF-α were detected in TG showing that the training induced an inflammatory process.

Also of note is that GAG depletion alone does not lead directly to increased cell death in the absence of abnormal stress stimuli[48]; this fact supports the hypothesis that even an exercise training regimen that gradually increases the training load to reach a strenuous running level can negatively affect the cartilage and eventually cause osteoarthritis.

The TG exhibited a lower concentration of HA compared to the CG, indicating that the strenuous running stimulated HA catabolism, consistent with recent work demonstrating that gene expression of hyaluronidases is increased by excessive mechanical stimulation and is also influenced by IL-1β[49]. The lower amounts of HA decreases lubrication and cartilage hydrophilicity, hindering its function as a molecular sponge and therefore its ability to withstand compressive forces; this hindrance weakens the already damaged tissue and increases its susceptibility to reinjury[3,49,50].

Conclusion

The articular cartilage of the rats subjected to gradual strenuous running regimen of controlled intensity exhibited significant predisposing osteoarthritic changes, including histopathological alterations, such as increased superficial fibrillation, clusters of chondrocytes and increased cell death; moreover it was observed a significant reduction of extracellular matrix components, such as sulfated GAGs and HA.

Animal study compliance

The research protocol (0302/08) was approved by the Animal Care Committee of the Federal University of São Paulo, according to the Brazilian Law number 11794/2008, for the use of animals in scientific academic research.

Contributions

All authors contributed to the research project's design and conception; data analysis and interpretation; and in the writing, revising and final approval of the manuscript.

Role of the funding source

Funding was provided by Brazilian funding agencies FAPESP, CAPES and CNPq.

Conflict of interest

None of the authors have conflicts of interest related to the manuscript.

Acknowledgments

The authors would like to thank the technical assistance of E. Kobayashi, M. Sc. and A. Mendes, M.Sc. from the Department of Biochemistry of Universidade Federal de São Paulo.

Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.joca.2013.04.007.

References

1. Duncan MJ, Spence JC, Mummery WK. Perceived environment and physical activity: a meta-analysis of selected environmental characteristics. Int J Behav Nutr Phys Act 2005;2:11.
2. Priest N, Armstrong R, Doyle J, Waters E. Interventions implemented through sporting organisations for increasing participation in sport. Cochrane Database Syst Rev 2008:CD004812.
3. Martel-Pelletier J, Boileau C, Pelletier JP, Roughley PJ. Cartilage in normal and osteoarthritis conditions. Best Pract Res Clin Rheumatol 2008;22:351–84.
4. Carter DR, Beaupré GS, Wong M, Smith RL, Andriacchi TP, Schurman DJ. The mechanobiology of articular cartilage development and degeneration. Clin Orthop Relat Res 2004 Oct;(427 Suppl):S69–77.
5. Lohmander S. Proteoglycans of joint cartilage. Structure, function, turnover and role as markers of joint disease. Baillieres Clin Rheumatol 1988;2:37–62.
6. Kim SH, Turnbull J, Guimond S. Extracellular matrix and cell signalling: the dynamic cooperation of integrin, proteoglycan and growth factor receptor. J Endocrinol 2011 May;209(2):139–51, http://dx.doi.org/10.1530/JOE-10-0377.
7. Gentili C, Cancedda R. Cartilage and bone extracellular matrix. Curr Pharm Des 2009;15:1334–48.
8. Kim HA, Blanco FJ. Cell death and apoptosis in osteoarthritic cartilage. Curr Drug Targets 2007;8:333–45.
9. Struglics A, Hansson M, Lohmander LS. Human aggrecanase generated synovial fluid fragment levels are elevated directly after knee injuries due to proteolysis both in the inter globular and chondroitin sulfate domains. Osteoarthritis Cartilage 2011;19:1047–57.
10. Bondeson J, Wainwright SD, Lauder S, Amos N, Hughes CE. The role of synovial macrophages and macrophage-produced cytokines in driving aggrecanases, matrix metalloproteinases, and other destructive and inflammatory responses in osteoarthritis. Arthritis Res Ther 2006;8:R187.
11. Helmark IC, Petersen MC, Christensen HE, Kjaer M, Langberg H. Moderate loading of the human osteoarthritic knee joint leads to lowering of intraarticular cartilage oligomeric matrix protein. Rheumatol Int 2011;32:1009–14.
12. Marti B, Knobloch M, Tschopp A, Jucker A, Howald H. Is excessive running predictive of degenerative hip disease? Controlled study of former elite athletes. BMJ 1989;299:91–3.
13. Roos EM, Dahlberg L. Positive effects of moderate exercise on glycosaminoglycan content in knee cartilage: a four-month, randomized, controlled trial in patients at risk of osteoarthritis. Arthritis Rheum 2005;52:3507–14.
14. Chakravarty EF, Hubert HB, Lingala VB, Zatarain E, Fries JF. Long distance running and knee osteoarthritis. A prospective study. Am J Prev Med 2008;35:133–8.
15. Noakes TD, Norman RJ, Buck RH, Godlonton J, Stevenson K, Pittaway D. The incidence of hyponatremia during prolonged ultraendurance exercise. Med Sci Sports Exerc 1990;22:165–70.
16. Peters EM. Nutritional aspects in ultra-endurance exercise. Curr Opin Clin Nutr Metab Care 2003;6:427–34.
17. Tarini VA, Carnevali Jr LC, Arida RM, Cunha CA, Alves ES, Seeleander MC, *et al.* Effect of exhaustive ultra-endurance exercise in muscular glycogen and both Alpha1 and Alpha2 Ampk protein expression in trained rats. J Physiol Biochem 2012, http://dx.doi.org/10.1007/s13105-012-0224-5.
18. Kilkenny C, Browne WJ, Cuthill IC, Emerson M, Altman DG. Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. Osteoarthritis Cartilage 2012;20:256–60.

19. Dishman RK, Armstrong RB, Delp MD, Graham RE, Dunn AL. Open-field behavior is not related to treadmill performance in exercising rats. Physiol Behav 1988;43:541–6.
20. Pritzker KP, Gay S, Jimenez SA, Ostergaard K, Pelletier JP, Revell PA, *et al*. Osteoarthritis cartilage histopathology: grading and staging. Osteoarthritis Cartilage 2006;14:13–29.
21. de Souza Merli LA, de Medeiros VP, Toma L, Reginato RD, Katchburian E, Nader HB, *et al*. The low level laser therapy effect on the remodeling of bone extracellular matrix. Photochem Photobiol 2012;88:1293–301.
22. Teixeira Gomes RC, Verna C, Nader HB, dos Santos Simoes R, Dreyfuss JL, Martins JR, *et al*. Concentration and distribution of hyaluronic acid in mouse uterus throughout the estrous cycle. Fertil Steril 2009;92:785–92.
23. Dietrich CP, McDuffie NM, Sampaio LO. Identification of acidic mucopolysaccharides by agarose gel electrophoresis. J Chromatogr 1977;130:299–304.
24. Beckwée D, Vaes P, Cnudde M, Swinnen E, Bautmans I. Osteoarthritis of the knee: why does exercise work? A qualitative study of the literature. Ageing Res Rev 2013 Jan;12(1):226–36.
25. Krampla WW, Newrkla SP, Kroener AH, Hruby WF. Changes on magnetic resonance tomography in the knee joints of marathon runners: a 10-year longitudinal study. Skeletal Radiol 2008;37:619–26.
26. Cheng Y, Macera CA, Davis DR, Ainsworth BE, Troped PJ, Blair SN. Physical activity and self-reported, physician-diagnosed osteoarthritis: is physical activity a risk factor? J Clin Epidemiol 2000;53:315–22.
27. Schueller-Weidekamm C, Schueller G, Uffmann M, Bader T. Incidence of chronic knee lesions in long-distance runners based on training level: findings at MRI. Eur J Radiol 2006;58:286–93.
28. Stehling C, Lane NE, Nevitt MC, Lynch J, McCulloch CE, Link TM. Subjects with higher physical activity levels have more severe focal knee lesions diagnosed with 3T MRI: analysis of a non-symptomatic cohort of the osteoarthritis initiative. Osteoarthritis Cartilage 2010;18:776–86.
29. Pap G, Eberhardt R, Sturmer I, Machner A, Schwarzberg H, Roessner A, *et al*. Development of osteoarthritis in the knee joints of Wistar rats after strenuous running exercise in a running wheel by intracranial self-stimulation. Pathol Res Pract 1998;194:41–7.
30. Chockalingam PS, Sun W, Rivera-Bermudez MA, Zeng W, Dufield DR, Larsson S, *et al*. Elevated aggrecanase activity in a rat model of joint injury is attenuated by an aggrecanase specific inhibitor. Osteoarthritis Cartilage 2010;19:315–23.
31. Fu SC, Cheuk YC, Hung LK, Chan KM. Limb Idleness Index (LII): a novel measurement of pain in a rat model of osteoarthritis. Osteoarthritis Cartilage 2012;20:1409–16.
32. Tang T, Muneta T, Ju YJ, Nimura A, Miyazaki K, Masuda H, *et al*. Serum keratan sulfate transiently increases in the early stage of osteoarthritis during strenuous running of rats: protective effect of intraarticular hyaluronan injection. Arthritis Res Ther 2008;10:R13.
33. Sekiya I, Tang T, Hayashi M, Morito T, Ju YJ, Mochizuki T, *et al*. Periodic knee injections of BMP-7 delay cartilage degeneration induced by excessive running in rats. J Orthop Res 2009;27:1088–92.
34. Poole R, Blake S, Buschmann M, Goldring S, Laverty S, Lockwood S, *et al*. Recommendations for the use of preclinical models in the study and treatment of osteoarthritis. Osteoarthritis Cartilage 2010;18(Suppl 3):S10–6.
35. Ikenoue T, Trindade MC, Lee MS, Lin EY, Schurman DJ, Goodman SB, *et al*. Mechanoregulation of human articular chondrocyte aggrecan and type II collagen expression by intermittent hydrostatic pressure in vitro. J Orthop Res 2003;21:110–6.
36. Blain EJ. Mechanical regulation of matrix metalloproteinases. Front Biosci 2007;12:507–27.
37. Poole CA. Articular cartilage chondrons: form, function and failure. J Anat 1997;191(Pt 1):1–13.
38. Lark MW, Bayne EK, Flanagan J, Harper CF, Hoerrner LA, Hutchinson NI, *et al*. Aggrecan degradation in human cartilage. Evidence for both matrix metalloproteinase and aggrecanase activity in normal, osteoarthritic, and rheumatoid joints. J Clin Invest 1997;100:93–106.
39. Almonte-Becerril M, Navarro-Garcia F, Gonzalez-Robles A, Vega-Lopez MA, Lavalle C, Kouri JB. Cell death of chondrocytes is a combination between apoptosis and autophagy during the pathogenesis of osteoarthritis within an experimental model. Apoptosis 2010;15:631–8.
40. Scott D, Smith C, Lohmander S, Chard J. Osteoarthritis. Clin Evid 2003 Dec;(10):1402–30.
41. Kobayashi M, Squires GR, Mousa A, Tanzer M, Zukor DJ, Antoniou J, *et al*. Role of interleukin-1 and tumor necrosis factor alpha in matrix degradation of human osteoarthritic cartilage. Arthritis Rheum 2005;52:128–35.
42. Kamiya T, Tanimoto K, Tanne Y, Lin YY, Kunimatsu R, Yoshioka M, *et al*. Effects of mechanical stimuli on the synthesis of superficial zone protein in chondrocytes. J Biomed Mater Res A 2010;92:801–5.
43. Lawyer T, Wingerter S, Tucci M, Benghuzzi H. Cellular effects of catabolic inflammatory cytokines on chondrocytes – biomed 2011. Biomed Sci Instrum 2011;47:252–7.
44. Struglics A, Larsson S, Pratta MA, Kumar S, Lark MW, Lohmander LS. Human osteoarthritis synovial fluid and joint cartilage contain both aggrecanase- and matrix metalloprot einase-generated aggrecan fragments. Osteoarthritis Cartilage 2006;14:101–13.
45. Thomas CM, Fuller CJ, Whittles CE, Sharif M. Chondrocyte death by apoptosis is associated with cartilage matrix degradation. Osteoarthritis Cartilage 2007;15:27–34.
46. Silver FH, Bradica G, Tria A. Do changes in the mechanical properties of articular cartilage promote catabolic destruction of cartilage and osteoarthritis? Matrix Biol 2004;23:467–76.
47. Rosenzweig DH, Djap MJ, Ou SJ, Quinn TM. Mechanical injury of bovine cartilage explants induces depth-dependent, transient changes in MAP kinase activity associated with apoptosis. Osteoarthritis Cartilage 2012;20:1591–602.
48. Otsuki S, Brinson DC, Creighton L, Kinoshita M, Sah RL, D'Lima D, *et al*. The effect of glycosaminoglycan loss on chondrocyte viability: a study on porcine cartilage explants. Arthritis Rheum 2008;58:1076–85.
49. Tanimoto K, Kitamura R, Tanne Y, Kamiya T, Kunimatsu R, Yoshioka M, *et al*. Modulation of hyaluronan catabolism in chondrocytes by mechanical stimuli. J Biomed Mater Res A 2010;93:373–80.
50. Bell CJ, Ingham E, Fisher J. Influence of hyaluronic acid on the time-dependent friction response of articular cartilage under different conditions. Proc Inst Mech Eng H 2006;220:23–31.